Ectopic hypermethylation of flower-specific genes in *Arabidopsis*

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Background: *Arabidopsis* mutations causing genome-wide hypomethylation are viable but display a number of specific developmental abnormalities, including some that resemble known floral homeotic mutations. We previously showed that one of the developmental abnormalities present in an antisense-*METHYLTRANSFERASEI* (*METI*) transgenic line resulted from ectopic hypermethylation of the *SUPERMAN* gene.

Results: Here, we investigate the extent to which hypermethylation of *SUPERMAN* occurs in several hypomethylation mutants, and describe methylation effects at a second gene, *AGAMOUS*. *SUPERMAN* gene hypermethylation occurred at a high frequency in several mutants that cause overall decreases in genomic DNA methylation. The hypermethylation pattern was largely similar in the different mutant backgrounds. Genetic analysis suggests that hypermethylation most likely arose either during meiosis or somatically in small sectors of the plant. A second floral development gene, *AGAMOUS*, also became hypermethylated and silenced in an *Arabidopsis* antisense-*METI* line.

Conclusions: These results suggest that ectopic hypermethylation of specific genes in mutant backgrounds that show overall decreases in methylation may be a widespread phenomenon that could explain many of the developmental defects seen in *Arabidopsis* methylation mutants. This resembles a phenomenon seen in cancer cells, which can simultaneously show genome-wide hypomethylation and hypermethylation of specific genes. Comparison of the methylated sequences in *SUPERMAN* and *AGAMOUS* suggests that hypermethylation could involve DNA secondary structures formed by pyrimidine-rich sequences.

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Background

Cytosine methylation is a reversible modification of DNA that plays a role in cellular memory in many eukaryotic taxa. It is associated with several epigenetic gene regulatory systems, including parental genomic imprinting, X-chromosome inactivation, and the silencing of transposons and other multiple-copy DNAs. Methylation also plays a role in tumor cell biology, as tumors often show both genome-wide demethylation and hypo- and hypermethylation of specific genes [1,2].

Studies of eukaryotic DNA methylation mutants have provided the opportunity to test the importance of methylation in a number of developmental processes. In mice containing targeted mutations of the methyltransferase *Dnmt1*, total DNA methylation is reduced to 30% of wild-type levels and embryos fail to differentiate and die after 9 days [3]. These mouse knock-out lines were used to show that proper levels of methyltransferase activity are required for allele-specific methylation and normal regulation of imprinted genes [4] and of the *Xist* gene [5]. Similar experiments were performed in *Arabidopsis* by expressing an antisense *METI* RNA to reduce the endogenous expression of the *Arabidopsis Dnmt1* homolog, *METI*. In contrast to the situation in mouse, the antisense-*METI* lines are viable, but display many specific and heritable developmental abnormalities including some defects in the flowers that resemble those of known floral homeotic mutants [6,7].

Arabidopsis plants homozygous for recessive mutations at either of two loci, *DECREASED DNA METHYLATION1* (*DDM1*) or *DDM2* (Eric Richards, personal communication), have a reduced overall level of cytosine methylation and display some of the same developmental defects as seen in the antisense-*METI* lines [8,9]. These developmental abnormalities map to discrete loci, different from the methylation loci, suggesting that alterations in methylation affect single genes that are important in development [9,10]. The *DDM1* locus has recently been cloned and shown to encode a putative SWI2/SNF2 class chromatin remodeling protein [11]. Thus, maintenance of proper methylation patterning in *Arabidopsis* may involve the coupling of DNA methyltransferase activity to chromatin remodeling. Mutations in *ddm2* map near the *METI*

methyltransferase gene [12] but conclusive evidence that *DDM2* and *METI* are identical has not been reported.

We previously described the molecular basis for one of the developmental defects observed in an antisense-*METI* line [13]. Surprisingly, this work showed that the floral development gene *SUPERMAN* (*SUP*) was ectopically hypermethylated and silenced, even though this antisense-*METI* line shows an overall decrease in genomic methylation of 80–90% [6]. The *SUP* gene was also found to be hypermethylated and silenced in seven independently isolated epigenetic *sup* alleles, originally called the *clark kent* alleles (*clk-1* to *-7*) and here designated the *sup^hm^* (*sup^hypermethylated^-1* to *-7*) alleles [13]. The *sup^hm^* lines all showed normal overall levels of genomic methylation [13]. In this report, we further investigate the effect of *Arabidopsis* methylation mutants on the hypermethylation of the *SUPERMAN* locus. We also demonstrate that another floral regulatory gene, *AGAMOUS*, also becomes hypermethylated.

Results

To test whether maintenance of the dense cytosine methylation present at the *SUP* locus might require wild-type activity of *DDM1* or *DDM2*, we crossed *sup^hm^-3 gl1-1* double mutant plants [13] with plants heterozygous for either *ddm1-2* or *ddm2-1* mutations. The *gl1-1* mutation eliminates epidermal hairs and maps roughly 10 centimorgans (cM) from *SUP* [14]. In the F2 populations of both of these crosses, plants with a *gl1-1* phenotype and reduced methylation at the centromeric repeat loci (a characteristic of *ddm* homozygosity [8]) were selected. Most of these plants displayed *sup^hm^-3*-like flowers. Bisulfite genomic sequencing confirmed that these plants were hypermethylated at *SUP*. Repeated self-pollination of these plants showed that *SUP* hypermethylation was stable in *ddm1* and *ddm2* homozygous backgrounds for at least three generations. Thus, neither *DDM1* nor *DDM2* activity is required for maintenance of *SUP* hypermethylation.

To test whether *SUP* hypermethylation and silencing can occur spontaneously in *ddm* mutants, *ddm1-2* and *ddm2-1* heterozygous plants were allowed to self-pollinate and several independent *ddm1-2* and *ddm2-1* homozygotes were analysed. These were then allowed to self-pollinate for several generations, and in each generation, an average of 50 plants were analysed. We found *sup*-like phenotypes coupled with *SUP* hypermethylation in most of the lines tested (Table 1). Some lines showed hypermethylation in the second generation after self-pollination, while others showed hypermethylation only after three or four selfed generations. In one case, hypermethylation of *SUP* was detected before the visible *sup* phenotype was seen (line B1, Table 1). Segregation analysis and complementation testing suggested that this was due to heterozygous hypermethylation of *SUP* in this line.

New *sup^hm^* alleles appeared in *ddm* homozygotes in a sporadic fashion; generally, only one or two plants of the 50 plants analysed showed *sup*-like phenotypes. This non-Mendelian inheritance suggests that the *ddm* homozygotes were chimeric for *SUP* hypermethylation. A clear demonstration of this is the *ddm2-1* homozygous line 176 (Table 1). All plants of this line had wild-type flowers in the second generation of self-pollination. When the first-generation homozygous plant was used as a male in a complementation cross to *sup-5* (a *sup* deletion mutant [13]), however, 1 out of 12 F1 plants showed a clear *sup* phenotype coupled with hypermethylation of *SUP*. Thus, plant 176 was a chimera, showing *SUP* hypermethylation in only a small proportion of its pollen, consistent with hypermethylation arising during meiosis or in small somatic sectors of the floral tissue.

We analysed the pattern of hypermethylation in a small region of the *SUP* locus in several different *ddm1* and *ddm2* homozygotes. The pattern of methylation was nearly identical in all of the lines tested (Figure 1). We detected only minor quantitative differences in the levels of methylation at each site. Furthermore, these differences were not reproducible, as we detected the same level of minor variation in replicate experiments with the same DNA samples as we did between the different mutant lines. The pattern in this region is also similar to that found previously in *sup^hm^-1* to *-7* and in an additional *sup^hm^* allele in an antisense-*METI* line [13]. Thus, regardless of the cause of hypermethylation at *SUP*, the methylation pattern within this region of the gene is consistent. We also found that the *SUP* locus becomes hypermethylated and silenced in two additional mutants that also cause overall genomic hypomethylation, *som5* and *som7*. These mutants were originally isolated based on their ability to derepress the activity of transcriptionally silenced transgenes [15], and were subsequently shown to be new alleles of *DDM1* [11]. Again, the pattern of hypermethylation of *SUP* was similar to that seen in the other genotypes.

The antisense-*METI* lines and the *ddm* mutants show a number of additional developmental abnormalities besides *superman*-like flowers [6,7,9]. One of the most striking phenotypes present in the antisense-*METI* lines is flowers that resemble those of the floral homeotic mutant *agamous* (*ag*). In *ag* mutants, stamens are converted to petals and the ovary to a new internal flower [16]. *AG* encodes a MADS-box protein and its RNA is expressed in the incipient and developing stamens and carpels [17]. The *ag* phenotype of antisense-*METI* plants is highly variable, with *AG*-like (wild-type for *AG*) and *ag*-like (similar to *ag* mutants) flowers occurring on the same plant. Figure 2a shows a wild-type flower (left, top), a flower from a strong loss-of-function *ag* mutant (right, top), and three flowers from an antisense-*METI* plant showing a range of *ag*-like defects (bottom). We have not observed similar *ag*-like phenotypes in the *ddm1* or *ddm2* mutants.

Table 1

Hypermethylation of *SUP* in *ddm* mutants.

Line name	Generation after self-pollination			
	1st	2nd	3rd	4th
ddm1-2 homozygotes				
B68	++, –, –	++, –, –	*sup*, Me, NC	
C4	++, –, –	++, –, –	++, Un, –	*sup*, Me, NC
204	++, Un, Co	++, –, –	*sup*, Me, –	
205	++, –, Co	*sup*, –, NC	*sup*, Me, –	
214	++, –, –	*sup*, –, –	*sup*, Me, –	
224	++, –, Co	++, –, –	++, –, –	
ddm2-1 homozygotes				
A2	++, –, –	++, –, –	++, –, –	*sup*, Me, –
B1	++, –, –	++, Me, NC*	*sup*, Me, NC	*sup*, Me, –
167	++, Un, Co	++, –, –	*sup*, Me, –	
170	++, –, Co	++, –, –	*sup*, Me, –	
176	++, –, NC†	++, –, –	++, –, –	++, –, –
178	++, –, Co	++, –, –	*sup*, Me, –	
180	++, –, Co	++, –, –	*sup*, Me, –	
181	++, –, Co	*sup*, –, –	*sup*, Me, –	
185	++, –, Co	++, –, –	++, –, –	*sup*, Me, –

The entries from left to right indicate: whether *sup*-like (*sup*) or wild-type (++) flowers were observed; whether hypermethylation of *SUP* was detected by bisulfite sequencing (Me) or the plants were tested and no hypermethylation was detected (Un); and whether the mutation complemented (Co) or failed to complement (NC) *sup-5*; –, not determined. *In the B1 × *sup-5* cross, 7 of 14 F1 plants were *sup*-like and showed hypermethylation. The selfed third generation progeny of line B1 segregated 3:1 wild-type:*sup*. Therefore, the second generation homozygote line B1 was most likely heterozygous for *SUP* hypermethylation. †In the 176 × *sup-5* cross, 1 of 12 F1 plants was *sup*-like and showed hypermethylation.

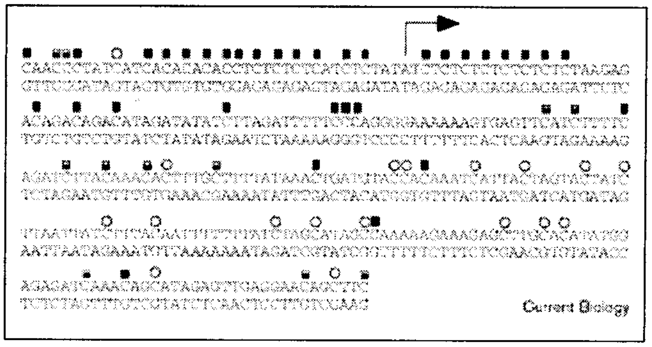
We sought to determine the molecular basis for the *ag*-like phenotype in the antisense-*METI* line. First, we showed that the *ag*-like flowers in the antisense-*METI* line had a reduced level of *AG* RNA, as judged by *in situ* hybridization (Figure 2b). Second, using bisulfite genomic sequencing, we found that *AG* was methylated in the antisense-*METI* line but not methylated in the wild type (Figure 3). We detected methylation in two regions of *AG*, the promoter and the large second intron. We assayed the methylation status of these two regions from either *ag*-like or *AG*-like flowers taken from a single antisense-*METI* plant. We found that the methylation in the promoter region was present in both types of flowers, but the methylation in the intron occurred exclusively in the *ag*-like flowers.

The composition of methylated sites at *AG* in the antisense-*METI* line was similar to that seen previously at *SUP* (Table 2; Figure 4) [13]. Most of the methylation was found at non-symmetric sites (sites other than CpG and CpXpG). Though there was little sequence-context specificity, there was some bias in favor of methylation at Cp(A/T)pG trinucleotides and a bias against methylation at CpC dinucleotides (Table 2). Methylation at CpG sites was not important for silencing of *AG*, as there were no CpG sites found in either the methylated promoter region or the methylated intron region. As an earlier report showed that *METI* is important for CpG methylation [6], these data suggest that, in the antisense-*METI* background, another methylase with a different specificity rather than hyperactivity of residual *METI* catalyses the hypermethylation of *AG*.

Methylation at *AG* was less dense than at *SUP*. In addition, none of the cytosines at the *AG* locus were fully methylated, as some are at the *SUP* locus (compare Figures 1,4). This may relate to the fact that the ag^{hm} phenotype is less stable than the sup^{hm} phenotype; the antisense-*METI* plants show a sup^{hm} phenotype throughout the entire body of the plant but they only show sectors with an ag^{hm} phenotype. The sectors of ag^{hm} flowers always developed on plants that already had the sup^{hm} phenotype, suggesting that hypermethylation of *SUP* occurs first and hypermethylation of *AG* may or may not follow.

To begin to understand the *cis*-acting signals that may cause these DNAs to be hypermethylated, we analysed the *SUP* and *AG* genomic sequences. Comparison of *SUP* and *AG* with several other *Arabidopsis* genes suggests that the DNA sequences are typical with respect to G + C content and in overall frequency of dinucleotides and trinucleotides (not shown). However, in the most densely methylated region of *SUP* (beginning at nucleotide –132 [18]), a pyrimidine-rich sequence containing many CT dinucleotides is present (5′-ATCACA-

Figure 1



Pattern of hypermethylation on the top strand of the *SUP* locus in *ddm1* and *ddm2* mutants. Because the data were obtained by direct sequencing of PCR-amplified genomic DNA, an average level of methylation was determined for each cytosine. Solid boxes indicate that more than 50% of the cytosines at this position were methylated, half-shaded boxes indicate that less than 50% of the cytosines were methylated, and open circles indicate that cytosine methylation was not detected. Arrow indicates the beginning of the *SUP* RNA [18]. Five independent *ddm1-2* plants (B68, C4, 204, 205 and 214) and eight independent *ddm2-1* plants (A2, B1, 167, 170, 176, 178, 180 and 181) showed a similar pattern. The 176 plant tested was an F1 from a cross between 176 and *sup-5*. All other plants were homozygous for *ddm1-2* or *ddm2-1*.

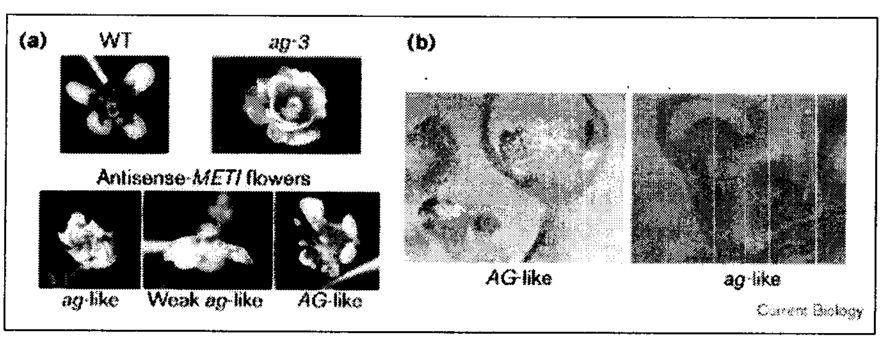
CACAC$(CT)_4$CAT$(CT)_2$ATAT$(CT)_8$A-3′). Methylation was detected at every C in this sequence [13]. Other pyrimidine-rich sequences were also found in both the promoter region and the intron region, which were methylated in *AG*. These are 5′-$(CT)_4$TTTTTTCTT-CATTTCC-3′ in the promoter region and 5′-$(CT)_3$-TTTTCTT$(CT)_2$TCTTT$(CT)_2$TACTTTCCTTTCT TAT$(CT)_2$AG$(CT)_3$TT$(CT)_3$C-3′ in the intron region (beginning at nucleotides 42890 and 45191 in GenBank sequence AL021711). These observations prompted us to test whether other similar sequences might also be methylated. To see how frequently these sequences occur in *Arabidopsis*, we used Blastn to search for sequences showing an exact match to 5′-TCTCTCTCTCTC-TCTCT-3′, which represents the longest CT-rich region in the methylated *SUP* sequence. As of the time of writing, a total of 874 database entries were found to contain this sequence out of a total of 97,935 sequence entries representing an estimated 81% of the complete *Arabidopsis* genome. This consisted of 137 hits within 45,752 expressed sequence tag entries, 118 hits within the 46,348 bacterial artificial chromosome end sequences, and 619 hits within the remaining 5,835 DNA entries. To test whether other such sequences are methylated in the antisense-*METI* lines, we analysed two additional sequences by bisulfite sequencing, one in the promoter region of *CARPEL FACTORY* [19], containing the sequence 5′-$(CT)_{22}$-3′ (beginning at nucleotide 22869 in GenBank sequence AC007323), and another near the beginning of transcription of the *LEAFY* gene [20] containing the sequence 5′-T$(CT)_6$ATCA$(CT)_3$TTTT$(CT)_3$-TTCTTTA$(CT)_2$-3′ (beginning at nucleotide 2742, GenBank sequence M91208). Methylation was not detected at either of these sequences. Thus, not all sequences that are rich in CT dinucleotides become hypermethylated in the antisense-*METI* plants, suggesting that some other aspect of the *SUP* and *AG* sequences is important.

Discussion

The observation that wild-type levels of *DDM1* or *DDM2* are not required for the establishment or maintenance of *SUP* hypermethylation contrasts sharply with results showing that methylation of the multi-copy *PAI* loci is abolished in a *ddm1* mutant background [21]. This suggests that the mechanism that maintains methylation at *SUP* may be different from the mechanism that maintains methylation at repetitive sequences throughout the genome. It is also interesting to note that *AG*, like *SUP*, occurs as a single copy in the *Arabidopsis* genome, again suggesting that this phenomenon may be somewhat different than repetitive gene methylation, as is seen for instance at the repetitive *PAI* loci [22], the rDNA loci, or the centromeric repeat loci [8].

SUP became hypermethylated at a high frequency in antisense-*METI* and in both *ddm1* and *ddm2* lines, and *AG*

Figure 2



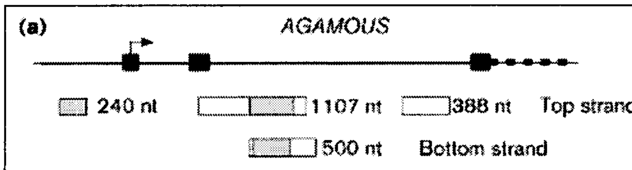
Antisense-*METI* flowers show a reduced expression level of the *AGAMOUS* gene. **(a)** Flowers of wild-type (WT) and *agamous-3* mutant flowers (top) and *agamous*-like and *AGAMOUS*-like flowers on antisense-*METI* plants (bottom). **(b)** Decreased *AG* RNA expression in antisense-*METI* flowers displaying *agamous*-like phenotypes. Flowers of the antisense-*METI* plants with either a wild-type *AG*-like phenotype or an *ag*-like phenotype were hybridized with a probe for the *AGAMOUS* gene. Yellow spots represent silver grains exposed by the ^{35}S-labeled probe after a 3 week exposure.

became hypermethylated in the antisense-*METI* line. This suggests that hypermethylation may be a common event in mutants showing overall hypomethylation. As some of the other epimutations seen in the various hypomethylation mutants are recessive [6,7,9], it seems likely that at least some of these may also be due to excess methylation of specific genes. This ectopic hypermethylation suggests that some aspect of methylation pattern fidelity is compromised when overall genomic methylation is decreased. One possible model to explain this is that factors that control the fidelity of genomic methylation are themselves regulated by DNA methylation. A second possibility is that residual DNA methyltransferase activity in these hypomethylation mutants is in some way hyperactivated when overall methylation is too low, resulting in ectopic methylation of some genes. It is not clear which DNA methyltransferases might be important for the establishment and/or maintenance of the methylation found at *SUP* and *AG*. Possible candidates include two new types of DNA methyltransferases that have been uncovered during the ongoing *Arabidopsis* genome sequencing efforts. The first are the chromomethylases first reported by Henikoff and Comai [23]. The first described gene, *CHROMOMETHYLASE1*, is disrupted by the insertion of a transposable element in the Ler ecotype and contains a splice-site mutation in the Columbia ecotype, indicating that the *CHROMOMETHYLASE1* gene is not required for hypermethylation of *SUP* in the *sup*^{hm}*-1* to -*7* lines (Ler) or in the *ddm* mutants (which originated in Columbia backgrounds and backcrossed into Ler [8]). At least one additional *CHROMOMETHYLASE* gene is present in the *Arabidopsis* genome (GenBank accession number AL021711); it appears from its sequence to be functional, and this could be important for methylation of *SUP* or *AG* [24]. A second new type of methyltransferase is represented by a family of *Arabidopsis* genes (X.C. and S.E.J., unpublished observations) showing similarity to the recently described mammalian Dnmt3 *de novo* methyltransferases [25]. Given the homology to *de novo* methylases, these are particularly good candidates for genes important in the establishment of *SUP* methylation.

We found that methylation of the second intron of *AG* correlated with the loss-of-function *ag* phenotype of anti-*METI*. This suggests that variable ectopic hypermethylation of this intron in somatic sectors of the plants causes variable levels of *AGAMOUS* gene silencing. These results are consistent with those of two studies

Figure 3

(a)



(b)

Genotype	Number of methyl-Cs detected: Promoter region	Intron 2
Antisense-*METI*		
ag-like	16	37
AG-like	16	0
Wild type C24	0	0

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Methylation detected in two regions of the *AG* locus in antisense-*METI* or wild-type plants. **(a)** The *AG* gene, showing exons (filled boxes), introns (lines) and the start of transcription (arrow). Open boxes below show regions where no methylation was detected; hatched boxes show regions where methylation was detected; nt, nucleotides. **(b)** Table of the number of methylated cytosines (methyl-Cs) in the promoter and intron region in the *ag*-like and *AG*-like flowers on the antisense-*METI* plants and in wild-type flowers of the C24 ecotype. All methylation-determination experiments were performed two independent times with two different plant samples, with the same result.

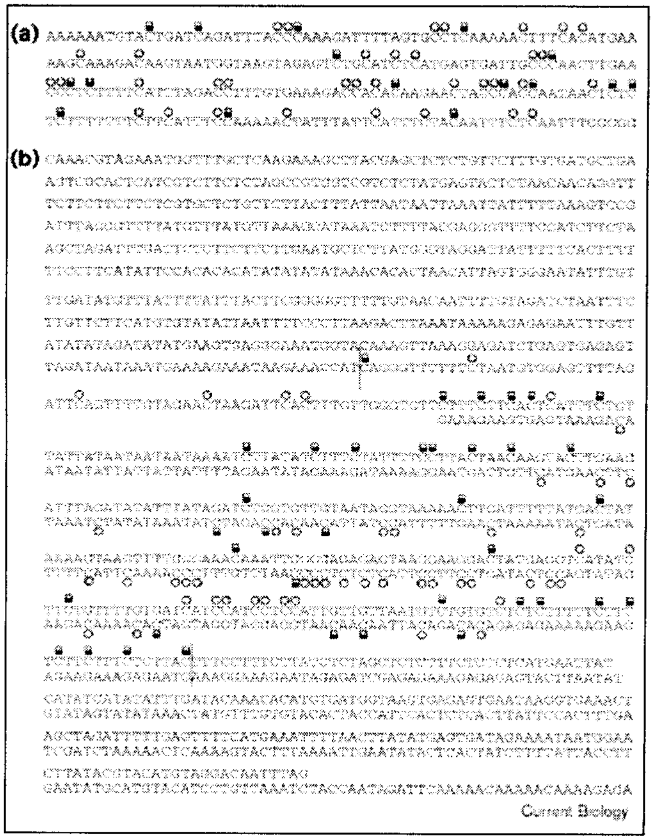
Table 2

Sequence context of methylated cytosines in *AGAMOUS* and *SUPERMAN* in antisense-*METI* plants.

AGAMOUS gene				*SUPERMAN* gene			
Sequence	Total number	Number methylated	Percentage methylated	Sequence	Total number	Number methylated	Percentage methylated
CX	129	53	41%	CX	383	186	49%
CA	46	20	43%	CA	129	75	58%
CT	56	32	57%	CT	156	88	56%
CG	0	–	–	CG	15	1	7%
CC	27	1	4%	CC	83	22	27%
CXA	40	18	45%	CXA	110	69	63%
CXT	46	15	33%	CXT	124	29	23%
CXG	12	10	83%	CXG	56	47	84%
CXC	31	10	32%	CXC	93	41	44%

AGAMOUS data were derived from the methylated regions shown in Figure 4. The cytosines included in this analysis are from the first methylated cytosine to the last methylated cytosine within the top strand of the methylated promoter region and the top and bottom strands of the intron region. *SUPERMAN* data are similarly derived from Figure 3b in reference [13].

Figure 4



Methylation pattern found at the *AGAMOUS* locus in anti-*METI* plants. **(a)** The *AGAMOUS* promoter region corresponding to positions 42715–42954 from GenBank sequence AL021711. **(b)** A region of the *AGAMOUS* second intron corresponding to positions 44305–45411 (top strand) and 44945–45444 (bottom strand) from GenBank sequence AL021711. Symbols are as in Figure 1. No methylation was found in the regions outside of the two vertical lines, and therefore no symbols are shown. An additional 388 nucleotide region of the second intron, 46675–47062 of GenBank sequence AL021711, was found to be completely unmethylated and is not shown.

showing that sequences necessary for proper *AG* RNA expression lie within this intron [26,27]. Busch *et al.* [27] have shown that a 3 kb fragment from this intron is sufficient to give a normal *AG*-like expression pattern when incorporated into reporter transgenes. Further dissection of this region shows that it is composed of at least two redundant enhancer elements, one consisting of the 5′ end of the intron and one consisting of the 3′ end of the intron, both of which are sufficient to produce a normal *AG*-like expression pattern during the early floral development [27]. We analysed the methylation pattern of both of these regions and found that the region toward the 5′ end was hypermethylated, but the region toward the 3′ end was not (Figures 3,4). It is unclear how hypermethylation of only one of these redundant enhancer elements could result in the observed loss of *AG* expression (Figure 2). One possibility is that the repressive effect of DNA methylation in this region spreads to affect the function of the entire intron.

It is not clear why particular sequences at the *SUP* and *AG* loci consistently became hypermethylated in the various methylation mutant lines. Analysis of the DNA sequences in the methylated regions show that they contain pyrimidine-rich sequences with an abundance of CT dinucleotides. The *Arabidopsis* genome contains many such sequences, however, and our analysis showed that two other CT-rich sequences present in the *CARPEL FACTORY* and *LEAFY* genes were unmethylated in the anti-*METI* line. One possibility is that, if these pyrimidine-rich sequences are involved in targeting *SUP* and *AG* for hypermethylation, such targeting might involve unusual DNA structures. Indeed, the pyrimidine-rich sequences in *SUP* and *AG* are predicted to form hairpin structures, whereas those in the unmethylated *CARPEL FACTORY* and *LEAFY* genes are not (see Materials and methods). It is also known that some polypyrimidine sequences participate in intramolecular triple-helix formation [28–30], which could further alter the secondary structure of these hairpins. In this light it is interesting to note that mammalian DNA methyltransferase has been shown to preferentially methylate hairpin structures *in vitro* as well as other unusual DNA structures [31–34]. Furthermore, inverted repeats appear to be particularly good targets for *de novo* methylation in *Arabidopsis* [35]. Transgenic experiments may be useful in identifying sequences that are necessary and sufficient for methylation of *SUP* and *AG*.

It is intriguing that two different floral regulatory genes have been found to be hypermethylated in the antisense-*METI* plants. Furthermore, other floral phenotypes are also present in the various methylation mutant lines including flowers that resemble *apetala1*, *apetala2* and *clavata* mutants [6] (S.E.J., unpublished observations). This raises the possibility that DNA methylation normally plays a role in the regulation of some floral development genes, and that the different methylation mutations cause misregulation of this system.

Conclusions

Our results demonstrate that the *SUPERMAN* locus becomes densely hypermethylated in several genetic backgrounds and that the *AGAMOUS* locus becomes hypermethylated in antisense-*METI*-containing plants. This suggests that hypermethylation of floral regulatory genes may be a common event in DNA hypomethylation mutants, and such hypermethylation could be the molecular basis for several additional floral abnormalities that are present in these lines. A detailed genetic understanding of this hypermethylation phenomenon should aid in our understanding of how proper methylation patterns are established and maintained in eukaryotic genomes.

Materials and methods

Plant strains and growth conditions

Seeds of *ddm1-2* and *ddm2-1* lines that had been extensively backcrossed into a wild-type Ler ecotype were a gift from Eric Richards. Seeds of the *som* mutants in the Zurich ecotype were a gift from Jurek Paszkowski and Ortrun Mittelsten Scheid. All plants were grown in constant illumination at 23°C. Plants were watered with a dilute solution of Miracle grow plant fertilizer (20:20:20).

Bisulfite genomic sequencing

Genomic DNA (2 µg) from the appropriate genotype was digested with restriction enzymes that cut just outside of the region of interest. The enzymes were *Eco*RI and *Fok*I for the *SUPERMAN* experiments or *Eco*RI and *Sac*I for *AGAMOUS*. Glycogen or 30 µg of tRNA was added, samples were extracted once with phenol/chloroform, and DNA was precipitated by adjusting to 3 M NH_4OAc pH 7.0, and adding three volumes of ethanol. After centrifugation, pellets were washed twice with 70% EtOH, dried, dissolved in 40 µl of water, heated at 97°C for 5 min and then quenched on ice. Freshly prepared 6.3 M NaOH (2 µl) was added and samples were incubated at 39°C for 30 min. Bisulfite solution (416 µl) was added to the denatured DNA and samples were overlaid with three drops of mineral oil. Samples were incubated in a thermal cycler for 5 cycles of 55°C for 3 h, 95°C for 5 min. The bisulfite solution was made by dissolving 40.5 g of sodium bisulfite (Fisher S654-500) in 80 ml of water with slow stirring to avoid aeration. The pH was adjusted to 5.1 with freshly prepared 10 M NaOH. Then, 3.3 ml of 20 mM hydroquinone was added (Sigma H-9003) and the volume was adjusted to 100 ml with water. After bisulfite conversion, the mineral oil was removed and samples were desalted with the Wizard DNA Clean-up System (Promega) following the manufacturer's instructions. NaOH was added to a final concentration of 0.3 M and samples were incubated at 37°C for 15 min. DNA was then precipitated by adding 2 µl of 20 µg/µl tRNA or glycogen, adjusting to 3 M NH_4OAc pH 7.0, and adding three volumes of ethanol. After centrifugation, pellets were washed with 70% ethanol and dissolved in 100 µl TE (10 mM Tris HCl, 1 mM EDTA pH 8.0). A 2 µl aliquot of this bisulfite treated DNA was used for each PCR reaction. Primer design and PCR conditions were similar to those previously described [36]. After purification of the PCR products on PCR quick spin columns (Qiagen), the PCR products were sequenced directly with the same primers used in the PCR or additional internal primers using ABI fluorescent sequencing.

In situ *hybridizations*

Longitudinal 8 µm sections of inflorescences were used for *in situ* hybridization experiments using an ^{35}S-labeled *AGAMOUS* probe as described in [18]. After a three-week exposure, slides were photographed using brightfield–darkfield double exposure.

Secondary structure predictions

The Stemloop program (GCG, Wisconsin package) was used to predict hairpin structures in the *SUP* and *AG* regions. The best hairpin structures that include the polypyrimidine sequences noted in the text are: SUP, 5′-TCTCTCTCATCTCTATATCTCTCTCTCTCTCT(C)TAAGAGACAGACAGACATAGATATATCTTAGA-3′; *AG* promoter region, 5′-TTTGTGAAAGACCACACAAGAACTACCCACC(A)ATAACTCTCTCTTTTTTTCTTCATTTCCAAA-3′; *AG* intron region, 5′-TTTCCTTTCTTATCTCTAGCTCTCTTTCTCTCTCATGAATTATAT(C)ATATCATATATTTGATACAAACACATGTGATGGTAAGTGAGAGTG-3′. The nucleotide in parentheses is the center of dyad symmetry. Using identical parameters, hairpins were not predicted to form in the pyrimidine-rich regions of *CARPEL FACTORY* or *LEAFY*.

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